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Regulation of Transcription of the Adenovirus EII Promoter by EIa Gene Products: Absence of Sequence Specificity

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During adenovirus infection, the EII promoter is positively regulated by products of the EIa region. We have studied this regulation by fusing a DNA segment containing the adenovirus EII promoter to a dihydrofolate reductase cDNA segment. Expression of this hybrid gene is stimulated in *trans* when cell lines containing an integrated copy are either transfected with plasmids carrying the EIa region or infected with adenovirus. This suggests that EIa activity regulates transcription of the EII promoter in the absence of other viral proteins and that this stimulation can occur when the EII promoter is organized in cellular chromatin. Transcription from the EII promoter is initiated at two sites in cell lines lacking EIa activity. Introduction of the EIa region preferentially stimulated transcription from one of these two sites. A sensitive, stable cotransfection assay was used to test for specific EII sequences required for stimulation. EIa activity stimulates all mutant promoters; the most extensive deletion retained only 18 base pairs of sequences upstream of the initiation site. We suggest that regulation of a promoter by the EIa region does not depend on the presence of a set of specific sequences, but instead reflects a characteristic of promoters that have been exogenously introduced into cells. Insertion of the 72-base-pair repeat of simian-virus 40 in *cis* enhances transcription from the EII promoter. The stimulatory effects of EIa activity and of the simian virus 40 sequence are additive and appear to differ mechanistically.

The EIa region of adenovirus encodes two interesting activities. First, this region positively regulates transcription from viral and cellular promoters (2, 8, 14, 23, 24, 33). Second, the region encodes proteins that can immortalize primary cells (14). The latter activity appears to be a critical component in the transformation of a normal cell to a tumor-forming cell and is an activity shared by a class of oncogenes that includes myc, myb, and the large tumor antigen of polyomavirus (18, 27, 30). It is not clear at this time whether the ability of the EIa products to regulate transcription is related to their ability to alter regulation of cellular growth. Determination of the mechanism by which the EIa region regulates transcription will help elucidate the relationship between these two activities.

The EIa region encodes three mRNAs of sizes 9S, 12S, and 13S (9, 26). The 289-amino-acid product of the 13S message stimulates expression of other adenovirus early transcription units by increasing the rate of transcription initiation (23, 28). Recently, it has been recognized that several non-adenoviral promoters are also stimulated by the EIa region (6a, 8, 33). In most positively regulated eucaryotic promoters, specific regulatory sequences have been identified which mediate this stimulation. For promoters stimulated by the EIa products, however, it has not been possible to separate sequences required for regulation from promoter sequences required for general transcription (3, 4, 8). These studies have been limited, though, by an inability to determine the effects of EIa activity on promoters weakened by deletion of sequences necessary for efficient transcription.

In previous studies, stimulation of transcription by the EIa products was observed 12 to 48 h after the affected promoter was introduced into the cell by either transfection or infection. The stimulated promoter was therefore present on extrachromosomal DNA. We have determined that a promoter stably integrated into cellular DNA can be stimulated by EIa activity. We have used this characteristic to develop a sensitive stable cotransfection assay and have investigated the ability of mutant promoters to respond to stimulation by the EIa region. The implications of these results are discussed in terms of the mechanism by which the EIa products stimulate transcription.

MATERIALS AND METHODS

Cell culture. The CHO DHFR-(DUKX BI) line has been described previously (34, 35) and was generously provided by L. Chasin. Cells were maintained in α^- special medium supplemented with adenosine (10 μg/ml), deoxyadenosine (10 μg/ml), thymidine (10 μg/ml), and 10% (vol/vol) fetal calf serum with penicillin and streptomycin (complete medium). Cells containing dihydrofolate reductase (DHFR) constructions were maintained in α^- media supplemented with 10% (vol/vol) dialyzed fetal calf serum and the indicated concentration of the drug methotrexate (MTX) (selective media).

DNA transfection and amplification. CHO DUKX BI cells were transfected with uncut plasmid DNA by the calcium phosphate coprecipitation technique of Graham and van der Eb (7). Cells were subcultured 20 h before transfection at a density of 10^6/10-cm dish. The indicated DNA was ethanol precipitated, dried, and suspended in 0.25 ml of 40 mM *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid (pH 7.05)–280 mM NaCl–40 mM KCl–2 mM Na_2HPO_4–0.2% (wt/vol) dextrose. The resultant DNA solution was rapidly mixed with 0.25 ml of 250 mM $CaCl_2$ by bubbling for 5 s followed by vortexing for 20 s. After 30 min at room temperature the precipitates were applied to the monolayers and incubated for another 30 min at room temperature. Subsequently, 4.5 ml of complete medium was added, and cells were incubated for 4 to 6 h at 37°C. Monolayers were then treated with 10% (vol/vol) glycerol in medium for 3 min, washed three times with medium and fed with complete

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medium. When cells reached confluence (2 days after transfection), they were subcultured at 1:15 in selective media with the indicated MTX concentrations. Cells were fed every 5 days. Ten days after transfection, all colonies were either stained and counted or picked and grown in selective media.

Transformants were amplified by selection in increasing amounts of MTX. Sequential increases involved steps of 0.02, 0.06, and 0.25 μM MTX.

Plasmids. Plasmid pEII-7, which contains the *Eco*RI F fragment of adenovirus type 2 (Ad2), was cleaved with *Eco*RI to completion followed by partial digestion with *Hae*III. The 400-base *Eco*RI (75.9 map units of Ad2 [6]-to-*Hae*III (74.8 map units of Ad2) fragment was ligated to the large fragment of pAdD26SV(A) (16) generated by *Eco*RI and *Bal*I cleavage. This ligation was done at 14°C for 1.5 h with 40 U of T4 DNA ligase to join the *Eco*RI cohesive termini. The resultant blunt-ended molecules were ligated to *Xho*I linkers, digested with an excess of *Xho*I, and circularized by ligation.

Plasmids in the pΔEII series were made by cleavage of pEII-7 with *Sma*I and digestion with the exonuclease BAL 31. *Eco*RI linkers were ligated to the termini, the DNA was cleaved with *Eco*RI and *Hin*dIII, and the large fragment was isolated after electrophoresis in low-melt agarose. The 29-base-pair (bp) *Eco*RI-*Hin*dIII fragment of pBR322 was ligated to this fragment to produce the pΔEII series. The endpoints of the deletions were determined by DNA sequencing (21). The endpoint of the deletion relative to the start site (+1) is indicated by the number in the plasmid name.

Plasmid pCVSVEII was made by inserting the EII promoter-DHFR cDNA segment spanning from *Sma*I to *Bam*HI of pΔEII-7 into *Pvu*II-*Bam*HI-cleaved pCVSVL (17).

Plasmid pHindIII G was constructed by K. Berkner and contains nucleotides 1 through 2,808 of Ad5 inserted in

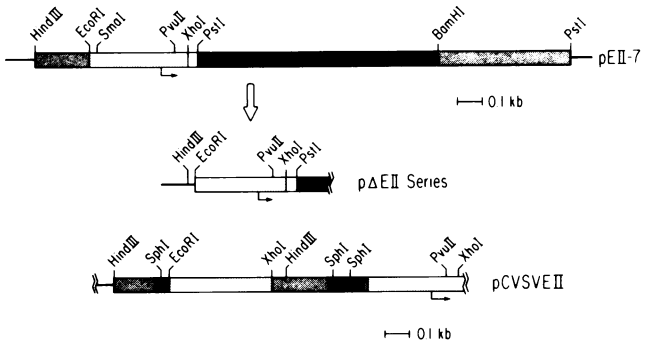

FIG. 1. EII-DHFR fusion vectors. All vectors contain the EII promoter fused to a DHFR cDNA. The *Hin*dIII-to-*Eco*RI fragment of pEII-7 contains nucleotides 5,171 to 160 of SV40; the *Eco*RI-to-*Xho*I fragment contains from 75.9% to 74.8% of Ad2 (6). The latter fragment includes the EII promoter start site and 5′ splice site. The remainder of pEII-7 has been described elsewhere (16) and contains a 3′ acceptor splice site, a mouse DHFR cDNA, and the SV40 early polyadenylation site. The pΔEII series plasmids were constructed as described in the text. The number after the dash indicates the number of wild-type bases remaining 5′ of the EII start site (site 1, Fig. 2). Plasmid pCVSVEII contains nucleotides 5,171 (*Hin*dIII) through 160 (*Eco*RI) of SV40, 14.7% (*Eco*RI) to 15.8% (*Xho*I) of Ad2, and nucleotides 5,118 (*Xho*I) through 270 of SV40 fused to the *Sma*I site of the EII promoter. The 72-bp enhancer regions are shown as filled boxes.

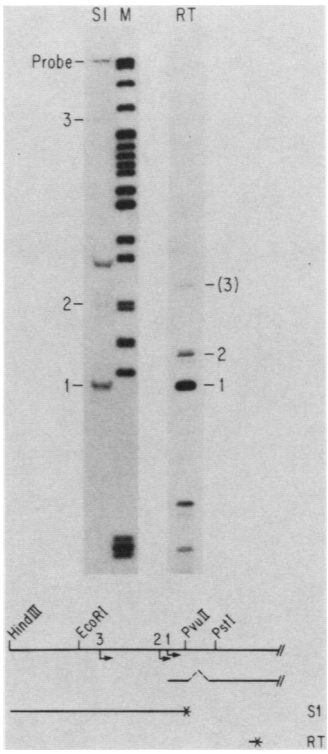

FIG. 2. Initiation sites for transcription from plasmid pEII-7. RNA from cell line 3AEII-7, which contains an integrated copy of plasmid pEII-7, was analyzed by nuclease S1 digestion or by primer extension. The S1-generated products have mobilities corresponding to 61 bases (site 1), 92 bases (site 2), and 280 bases (site 3) as compared with the *Hpa*II-cleaved pBR322 used as markers. The band at 110 bases is an artifact of the probe preparation used. The primer extended products migrate as 220 bases (site 1), 245 bases (site 2), and 330 bases (site 3) as compared with DNA markers (not shown; see Fig. 7). The 21-base primer used for reverse transcriptase analysis (a gift of K. Romachandran) has the sequence 5′-GAC GAT GCA GTT CAA TGG TCG-3′ and is homologous to the DHFR cDNA-coding region. Line 3AEII-7 was amplified to resistance to 15 μM MTX to facilitate analysis.

pBR322. Plasmid pGhr1-3 was constructed by removing the EIa-encoding *Xma*I-*Hin*dIII fragment of pHindIII G and replacing it with the same *Xma*I-*Hin*dIII fragment from phr1 (a gift of B. Roberts). phr1 contains the EIa region of the host range mutant Ad5 hr1 (10). Plasmid pEKpm975 (a gift of C. Montell and A. Berk) contains nucleotides 1 through 2,045 of Ad5 with a point mutation at position 975 that alters the splicing site necessary to form the EIa 12S message (22).

Analysis of transfected DNA. High-molecular-weight cellular DNA was isolated as described by Steffen et al. (32). DNA was digested with *Bam*HI, and 10 μg was separated on a 1.0% agarose gel. Separated DNA was transferred to nitrocellulose by the method of Southern (31). DNA fragments containing either the EIa region (nucleotides 1 through 2,808 of Ad5) or DHFR-coding sequences (the 1.45-kilobase *Eco*RI-*Bam*HI fragment of pEII-7) were purified by agarose gel electrophoresis and glass powder elution (36). These fragments were labeled with α^{-32}P-labeled nucleotide triphosphates by nick translation (29).

RNA analysis. Total cellular RNA was isolated by the method of Favaloro et al. (5). For primer extension analysis, the indicated amount of RNA was mixed with 3×10^5 cpm of

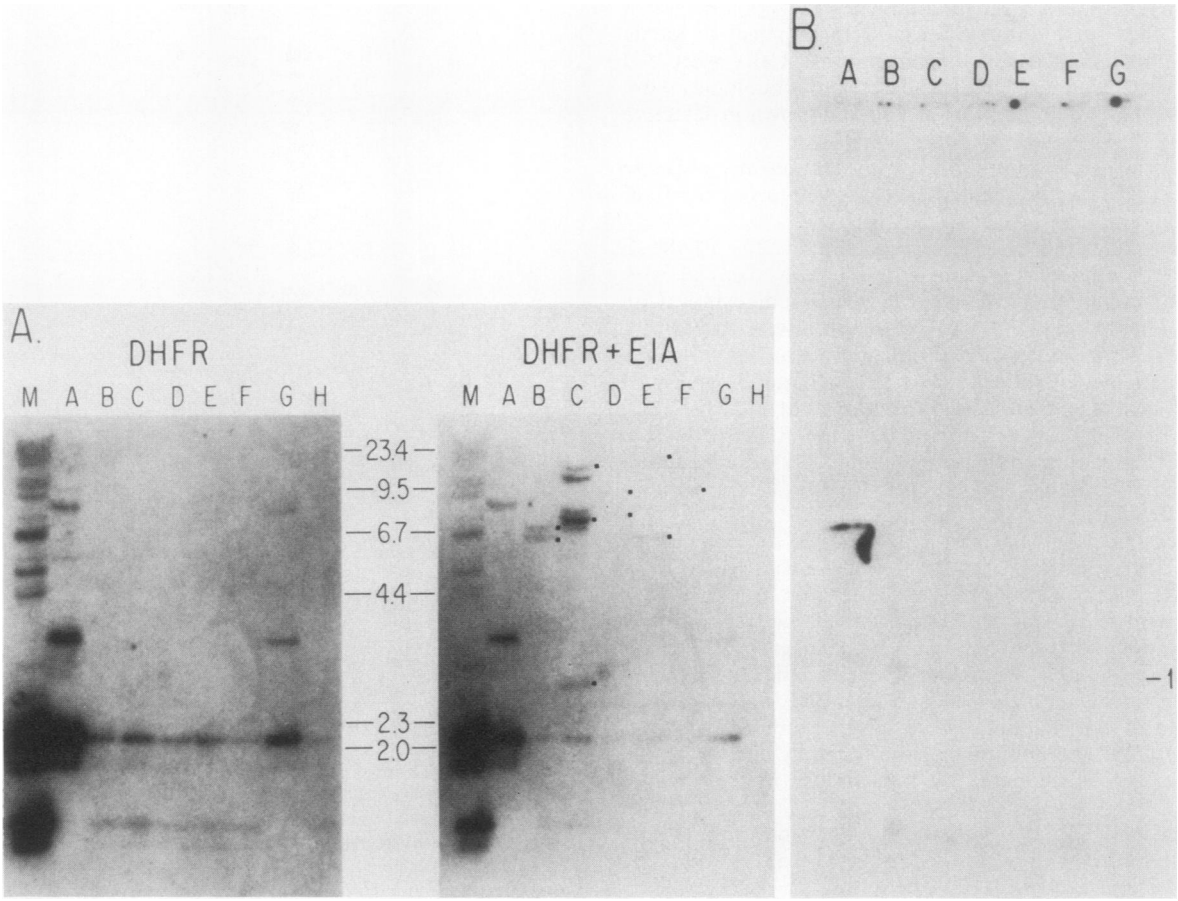

FIG. 3 Analysis of cell lines containing pEII-7 and EIa region DNA. (A) DNA from the indicated cell lines was digested with restriction enzyme *Bam*HI, electrophoresed, and transferred to nitrocellulose. *Bam*HI cleaves twice in plasmid pEII-7 to produce a 2.1-kilobase fragment containing the entire DHFR hybrid gene. The filter was hybridized with probe either complementary to DHFR sequences or complementary to both DHFR and EIa sequences, as indicated. The dots indicate fragments hybridizing to the EIa region. The cell lines in lanes A through G were all isolated from colonies growing at 0.02 μM MTX as described in the text. They are the result of transfection of cell line 3AEII-7 with the following DNA: lane A, control ($pSV\alpha_1p3d$); lanes B through G, pHindIIIG. Lane H contains 3AEII-7 DNA, and lane M is an independently transfected cell line used as a marker. The positions of the *Hin*dIII-cleaved λ DNA markers are shown. (B) Total RNA (20 μg) of the cell lines of panel A were analyzed by primer extension as described in Fig. 2. Lanes A through G correspond to lanes B through H, respectively, of panel A. The band labeled 1 comigrates with that labeled 1 in Fig. 2.

a 5′ end-labeled 21-base primer (see Fig. 2; synthesized by K. Ramachandran, Biogen) and incubated at 30°C for 16 h in 30 μl of hybridization solution [80% formamide–0.4 M NaCl–40 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (pH 6.4)–1 mM EDTA]. A 170-μl amount of 0.3 M sodium acetate was added, the RNA-DNA hybrids were ethanol precipitated, and the pellet was washed with 70% ethanol. The hybrids were suspended in 25 μl of buffer (50 mM Tris [pH 8.3]–40 mM KCl–8 mM $MgCl_2$–1mM dithiothreitol–0.4 mM each dATP, dGTP, dTTP, AND dCTP). Reverse transcriptase (15 to 20 U; Life Science, Inc.) and 15 Units of RNasin (Promega Biotec) were added, and the mixture was incubated for 1 h at 37°C. After primer extension, RNA was digested with pancreatic RNase, and DNA was separated on an 8.0% acrylamide gel (acrylamide/bisacrylamide ratio of 30:1) containing 7 M urea (19).

For S1 analysis, hybrids were made exactly as described above, and 300 μl of cocktail (250 mM NaCl–30 mM sodium acetate [pH 4.6]–1 mM $ZnSO_4$–5% glycerol–20 μg of calf thymus DNA per ml–1,000 U of S1 per ml) was added. The reaction was incubated for 2 h at 14°C, and the products were ethanol precipitated and analyzed as described above.

RESULTS

Construction and characterization of an EII-DHFR hybrid gene. To assay transcription from the adenovirus EII early promoter, we joined a 400-bp fragment containing the promoter to a DHFR cDNA gene (Fig. 1). This hybrid gene was active in transforming DHFR-deficient CHO cells to a prototrophic state. The structure of the DHFR mRNAs in the stably transformed cell lines was investigated both by S1 nuclease analysis and by primer extension. In the latter case, a 21-bp oligonucleotide homologous to DHFR cDNA was used as primer for elongation by reverse transcriptase (Fig. 2; see Fig. 7). Both methods demonstrated that the major DHFR mRNAs in these lines have 5′ termini at the position previously determined as the initiation site (site 1) for EII transcription during virus infection (Fig. 2) (1). A fraction of the DHFR mRNAs (start site in Fig. 2 and 7) had 5′ termini mapping 26 bases upstream of this initiation site at a position prominently recognized for transcription in vitro of the EII promoter (20). A third RNA (Fig. 2) initiates at a cryptic initiation site 200 bases upstream of site 1.

Stimulation of transcription from an integrated EII promot-

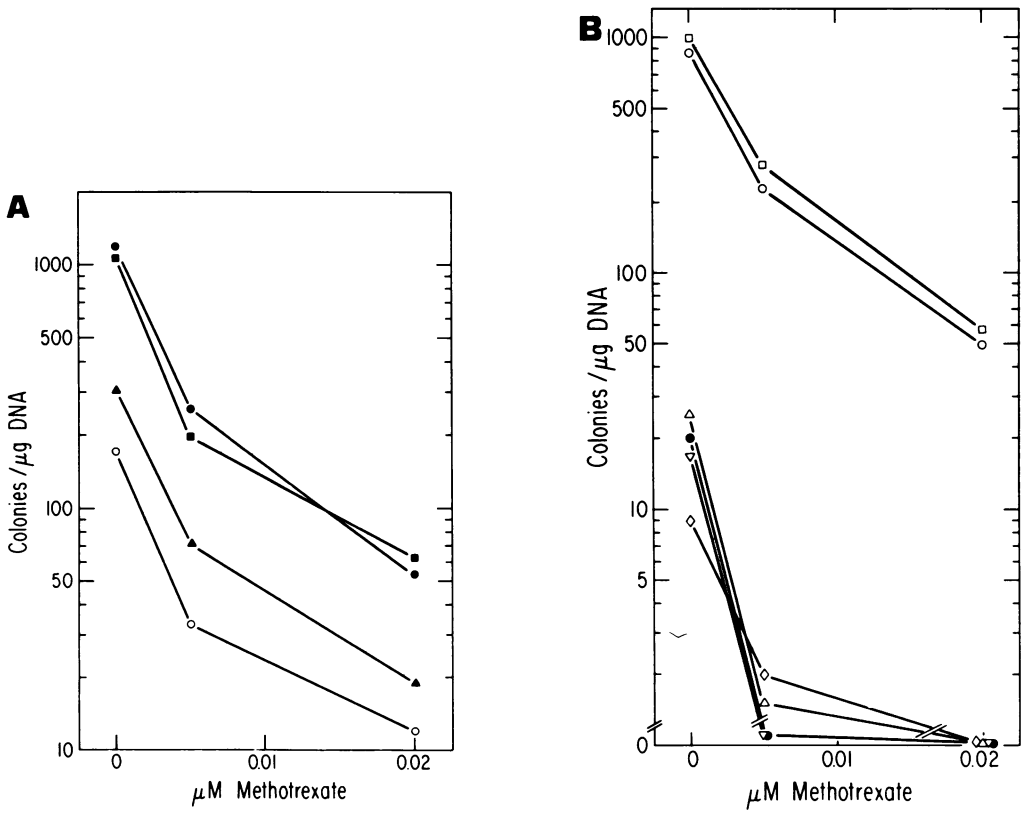

FIG. 4. Effect of cotransfection of pΔEII-260 with DNA containing wild-type and mutant EIa regions. Plasmid pΔEII-260 (1 μg) was mixed with either pBR322 DNA (5 μg; ○), pHindIII G DNA (5 μg, ■), pEKpm975 DNA (5 μg, ●), or pGhr1 DNA (5 μg, ▲) and transfected into CHO DUKX B1 cells. Colonies were counted 12 days after transfection. (B) Effects of deletions on transfection efficiency of pΔEII plasmids. CHO DUKX B1 cells were transfected with 6 μg of the indicated plasmid: pΔEII-260 (○), pΔEII-96 (□), pΔEII-57 (△), pΔEII-43 (◇), pΔEII-30 (▽), or pΔEII-18 (○). The ordinate values refer to the amount of DHFR-containing plasmid.

er. Transcription from the EII region peaks approximately 8 h after adenovirus infection (25). Virus carrying mutations in the EIa region produce a 100-fold lower level of EII mRNAs at the same stage of infection. During viral infection, the EII promoter enters the cell as virion DNA and is transiently stimulated for transcription in the presence of many viral genes. We wished to determine whether a stably integrated EII promoter region could respond in *trans* to EIa activity and whether stable integration of DNA containing the EIa genes would result in continual stimulation of EII transcription.

A cell line (3AEII-7) carrying 400 bp of the EII promoter joined to the DHFR cDNA segment was transfected either with a plasmid containing the Ad5 EIa region (pHindIIIG) or with an equivalent amount of control DNA. Transfected cells were split into media containing increasing amounts of MTX. MTX inhibits DHFR activity by binding to the enzyme, and thus cell growth in the presence of higher concentrations of the drug requires higher levels of DHFR. 3AEII-7 cells could potentially form colonies in the presence of increasing levels of MTX either by increased expression of a constant number of EII-DHFR genes or by amplification of the copy number of this gene. Transfection of these cells with plasmids containing the EIa region resulted in formation of 510 and 45 colonies per 10^6 cells at 0.02 and 0.05 μM MTX, respectively. This compared to 220 and 14 colonies after transfection with control DNA, suggesting that the integration of the EIa-containing DNA stimulated production of DHFR.

If the EIa genes were responsible for stimulation of the EII promoter, then colonies forming at higher MTX concentrations should contain EIa sequences. Analysis of the genomic DNA from six lines transfected with EIa DNA and capable of growth at 0.02 μM MTX revealed that five of these lines indeed contained integrated EIa DNA (lanes B through F, Fig. 3A; the bands labeled by dots hybridize to EIa sequences). These five lines contained similar levels of EII-DHFR DNA as the parental 3AEII-7 line. Only approximately 0.1% of cells transfected with DNA stably integrate that DNA. The observation that five of six lines growing at 0.02 μM MTX contain EIa sequences strongly implies that these sequences are responsible for the ability of the cells to grow under the selective conditions. The two lines that did not contain EIa DNA contained amplified levels of the integrated EII-DHFR sequences (lanes A and G, Fig. 3A). In all tested cases, the ability to grow at higher levels of MTX was correlated with an increase in DHFR mRNA levels (Fig. 3B). Thus, EIa acts continuously to increase the level of mRNA per copy of the integrated EII-DHFR genes.

Sequences in the EII promoter necessary for regulation by EIa. The above data strongly suggest that EIa activity can stimulate transcription in *trans* from a stably integrated EII promoter. To determine what sequences in the EII promoter allow response to EIa activity, a more rapid assay was developed (see also references 3 and 37). DHFR-deficient CHO cells were cotransfected with plasmids containing the EII-DHFR fusion gene and either pBR322 DNA or DNA containing the EIa region. Cotransfection with EIa plasmid

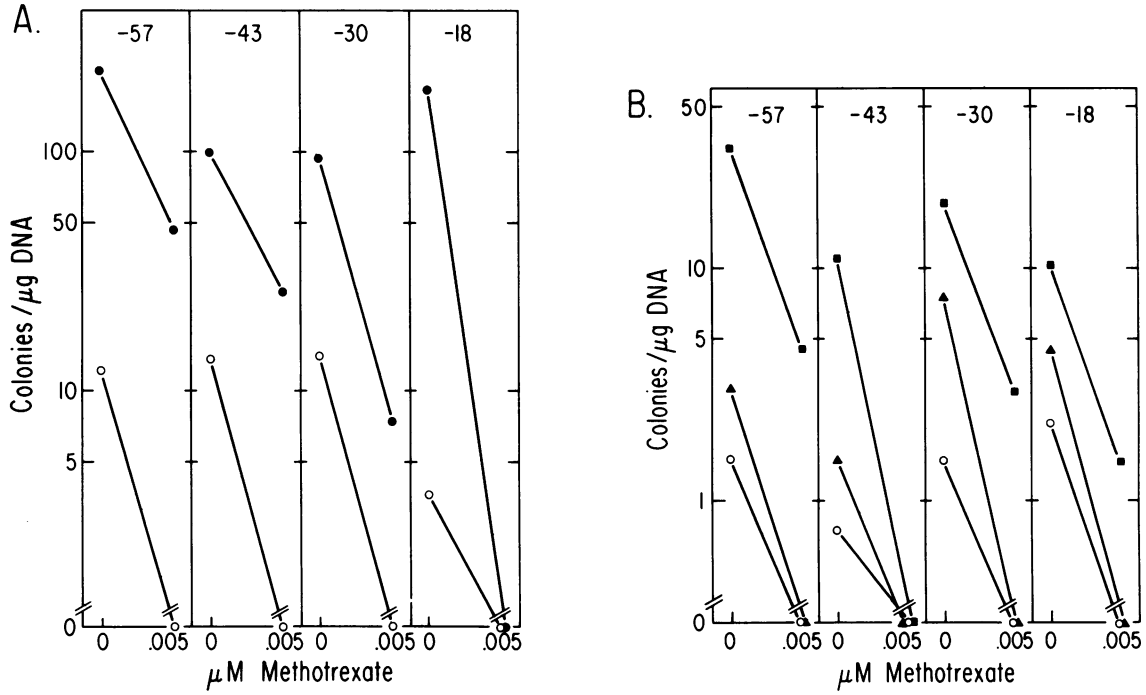

FIG. 5. Effect of EIa activity on expression from mutant EII promoters. (A) The indicated pΔEII series plasmid (2 μg) was mixed either with pBR322 (4 μg, ○) or with pBR322 (2 μg) and pEKpm975 DNA (2 μg, ●) and transfected into CHO DUKX B1 cells. (B) The indicated pΔEII series plasmid (2 μg,○) was mixed either with pBR322 (4 μg, ○), with pBR322 (2 μg) and pHindIII G (2 μg, ■), or with pBR322 (2 μg) and pGhr1 (2 μg, ▲) and transfected into CHO DUKX B1 cells. The experiments in panel A were done at a different time than those in panel B, and thus transfection efficiencies cannot be compared between the two panels. The ordinate values refer to the amount of DHFR-containing plasmid.

would be expected to yield an increase in the number of stable colonies capable of growth in selective media (media lacking adenosine, deoxyadenosine, and thymidine). In fact, such an increase was observed at three increasingly stringent levels of selection (0, 0.005, and 0.02 μM MTX; Fig. 4A).

DNA molecules introduced into a cell by transfection tend to recombine. We therefore felt it necessary to show that the observed stimulation by the EIa region was a result of an EIa gene product and not of any *cis*-acting transcriptional enhancer activity contained on the EIa plasmid (11, 25a). Plasmids containing two mutant EIa regions were tested in the stable cotransfection assay. Point mutant pm975 is defective for synthesis of the 12S EIa message (243-amino-acid protein), but produces a wild-type 13S message (289-amino-acid protein) (22). Virus with this mutation regulates EII transcription in a normal fashion during infection. Viral mutant Ad5 hr1 carries a frameshift mutation in the reading frame of the 13S message, but produces a wild-type 12S message (10, 28). Ad5 hr1 virus is impaired in its ability to stimulate EII transcription (2). In the stable cotransfection assay, both point mutants behaved analogously to their phenotype during infection. The pm975 mutant stimulates colony formation at wild-type levels; the hr1 mutant does not (Fig. 4A).

To test whether specific EII promoter sequences are necessary for regulation by EIa activity, we constructed a series of deletions that extended into the EII promoter from the 5′ side (Fig. 1). The ability of these deletions to stably transform DHFR-deficient CHO cells was compared (Fig. 4B). Deletions to −260 (numbered with the start site at +1) and −96 had identical activity in the assay. Deletions to −57, −43, −30, and −18 had significantly lower activities, suggesting that an important promoter sequence lies between −96 and −57. All deletions, including the deletion to −18, were positively regulated by the EIa region in the stable cotransfection assay (Fig. 5A). Thus, the stimulatory action of EIa did not depend on any specific EII promoter sequences upstream of −18. This regulation was dependent on synthesis of the 289-amino-acid protein from the EIa region, as cotransfection with a plasmid containing the point mutant hr1 resulted in the formation of fewer colonies than seen after cotransfection with the wild-type EIa region (Fig. 5B).

Effect of an enhancer sequence on regulation. Stimulation of transcription by the EIa region in *trans* and by "enhancer" regions in *cis* have superficial similarities. Both function on a wide variety of promoters, and neither appears to act through a specific promoter sequence. Furthermore, the addition of enhancer elements to some gene constructions that are stimulated by EIa activity can reduce or eliminate this stimulation (8; R. Kingston, unpublished observations). The 72-bp repeat enhancer element of simian virus 40 (SV40) was inserted at the 5′ side of the EII promoter (Fig. 1, pCVSVEII). As expected, pCVSVEII DNA shows an increased activity over that of the parental pΔEII-260 DNA (Fig. 6). This increased expression is due to initiation at the EII promoter, since DHFR mRNAs in pCVSVEII-transfected cells have 5′ termini in common with pEII-7- and pΔEII-260-transfected cells (Fig. 7, lanes A through D). This evidence suggests that transcription from the EII promoter in pCVSVEII is enhanced by the SV40 element.

When assayed by stable cotransfection, the expression of both the normal and enhanced EII promoter constructions was stimulated by EIa activity (Fig. 6). This result was corroborated by infecting cell lines containing integrated EII-DHFR fusions with Ad5. When RNA levels were ana-

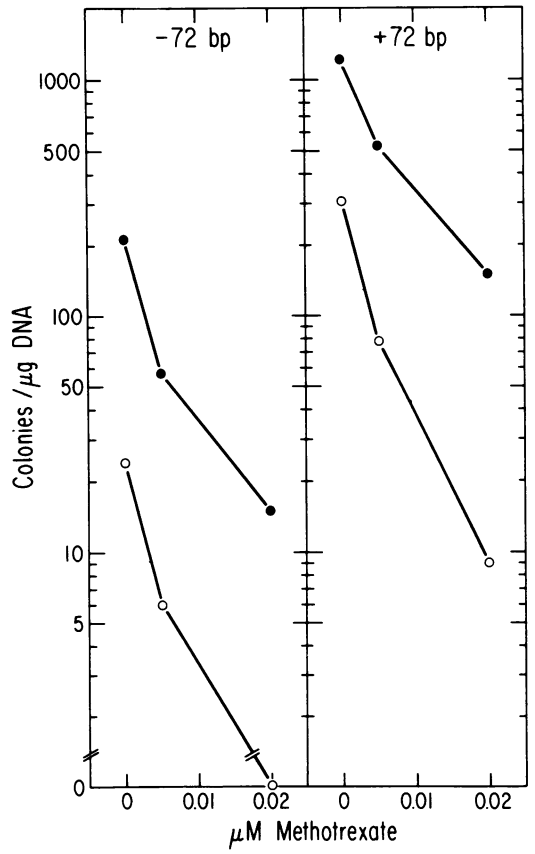

FIG. 6. Effects of EIa activity and an enhancer region on expression of the EII promoter. Plasmid pΔEII-260 (1 μg, −72 bp) or plasmid pCVSVEII (1 μg, +72 bp) was mixed with either pBR322 (5 μg, ○) or pEKpm975 (5 μg, ●) and transfected into CHO DUKX B1 cells.

lyzed 12 h after infection, the level of mRNA initiated at the prominent EII site in both promoters was increased (Fig. 7, lanes E through L). Interestingly, stimulation by EIa activity resulted in either no or very little increase in the level of mRNA initiated at the site 26 nucleotides upstream of the EII site (site 2, Fig. 7). This contrasts with the enhancement effect of the 72-bp region, which stimulated both initiation sites of the EII promoter equally.

DISCUSSION

The adenovirus EIa region positively regulates transcription from the EII promoter during the normal course of lytic infection (2, 14). We show here, in four separate cases, that this regulation can occur as well when the EII promoter region is stably integrated into the cellular genome (Fig. 3 and 7). EIa does not simply induce a transiently altered transcriptional state in the cell; introduction of a plasmid containing the EIa region constitutively increases the expression from the integrated EII promoter of cell line 3AEII-7 (Fig. 3). These data argue that no other viral gene products apart from the EIa region products are required for stimulation of transcription.

The EIa region of adenovirus regulates gene expression by increasing the efficiency of initiation at the affected promoter (23). We have investigated the mechanism by which initiation from the adenovirus EII promoter is increased. Cotransfection into CHO cells of plasmids containing the EIa region with plasmids containing promoters joined to a cDNA segment encoding DHFR and subsequent selection of stable colonies was used to analyze a series of deletions of the EII promoter. An EIa region containing a point mutation was defective for stimulation of colony formation in this cotransfection assay in a fashion equivalent to its phenotype during lytic infection. Deletion of a sequence between −57 and −96 lowers the activity of the EII promoter both in the presence and absence of the EIa products (see also reference 4). However, all deletions including the most extensive, which deletes all sequences upstream of −18 of the EII promoter, are positively regulated by the EIa gene product in this assay (Fig. 5). This suggests that there are no specific sequences

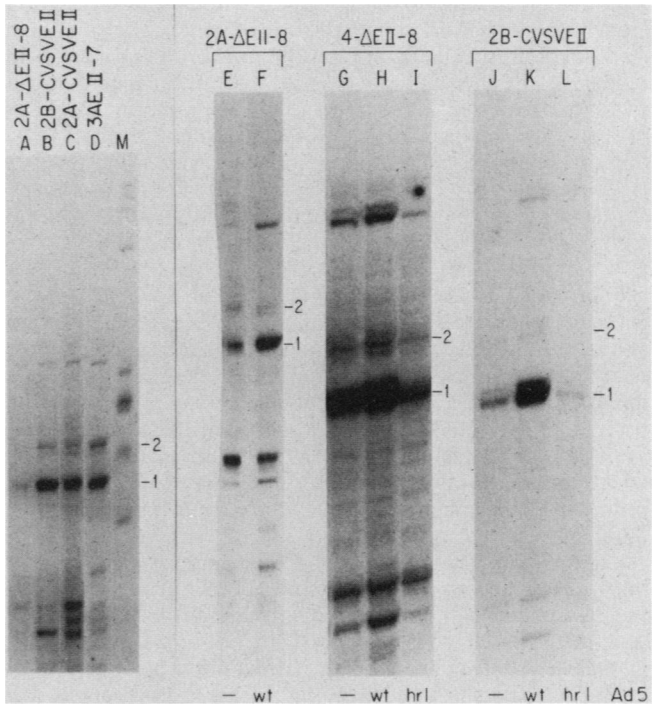

FIG. 7. Analysis of transcription level and initiation sites in cell lines carrying the EII promoter. Total RNA (40 μg, lanes A through D; 20 μg, lanes E and F; 10 μg, lanes G through L) was analyzed by primer extension of a 21-base primer (Fig. 2). Analyses are of cell lines containing the following plasmids stably integrated in genomic DNA: lanes A, E, F, and cell line 2A-ΔEII-8 (contains pΔEII-260); lanes B, J, K, and L, cell line 2B-CVSVEII (contains pCVSVEII); lane C, cell line 2A-CVSVEII (contains pCVSVEII); lane D, cell line 3AEII-7 (contains pEII-7); lanes G through I, cell line 4-ΔEII-8 (contains pΔEII-260). Lane M shows the *Hae*III cleaved ϕX174 DNA as size markers. RNA was isolated 12 h after infection of subconfluent monolayers of the indicated cell lines by Ad5. Lanes E, G, and J, Mock infection; lanes F, H, and K, Ad5 infected at a multiplicity of infection of 20; lanes I and L, Ad5 hrl infected at a multiplicity of infection of 20. The amount of RNA made from each start site was determined by laser densitometer scanning of the autoradiograms. The level of induction of each start site was as follows (expressed as the infected/uninfected ratio): lanes E and F, site 1 fourfold, site 2 onefold; lanes G and H, site 1 twofold, site 2 onefold; lanes J and K; site 1 fourfold, site 2 twofold. All cell lines contain amplified DHFR genes to facilitate analysis. Cell lines 4-ΔEII-8, 2A-CVSVEII, 2B-CVSVEII, and 3AEII-7 all grow at 0.25 μM MTX, cell line 2A-ΔEII-8 grows at 0.06 μM MTX.

either upstream of or in the EII promoter required for stimulation of transcription by EIa activity.

In addition to adenovirus early promoters, the human β-globin, SV40 early, and rat preproinsulin I promoters have all been shown to be positively regulated by EIa activity (8, 33; Gaynor et al., in press). Deletion studies on the β-globin and SV40 early promoter and on the adenovirus EIb promoter have failed to identify a sequence that is necessary for stimulation of these promoters by EIa activity (3, 8). In all of the cases studied, it is possible that sequences 3′ to the initiation site may determine the ability of a promoter to respond to EIa activity. However, the wide variety of promoters regulated by EIa and the inability to locate specific regulatory sequences within those promoters argue that the sequences required for positive regulation by EIa are a subset of those that define the promoter. No specific sequences appear to determine this regulation.

EIa stimulation and enhancers. The EIa activity shares with enhancer regions the ability to stimulate transcription from numerous promoters. Insertion of enhancer elements in a *cis* position can eliminate the ability of certain promoters to respond to EIa activity (34; R. Kingston, unpublished observations). However, in the case of the EII promoter, insertion of the 72-bp enhancer region of SV40 and cotransfection by the EIa region stimulate expression in an additive manner (Fig. 4). Another indication that stimulation of promoters by EIa activity and enhancer elements are different is apparent from analysis of the sites for the initiation of EII transcription. Initiation from the EII promoter occurs at two sites that are separated by 26 nucleotides (1, 20). The downstream site is the predominant site utilized in vivo whereas the upstream site is only utilized to produce a minor species under most conditions. Insertion of the enhancer element stimulates initiation at both EII sites equally, whereas the EIa region preferentially stimulates the downstream EII start site (Fig. 7). Thus, although enhancer elements and EIa must both affect the same rate-limiting step in expression of certain promoters, the mechanism by which each functions must differ.

EIa function. The EIa gene product can positively regulate in *trans* a wide variety of viral and cellular promoters when these promoters are introduced into cells either by transfection, microinjection, or infection (8, 15, 33, 37; Gaynor et al., in press). As discussed above, it has not been possible to identify a specific sequence within these promoters that determines their ability to be regulated. Transcription stimulation by EIa activity cannot be totally nonspecific, since not all transfected promoters are regulated by EIa activity; the herpes TK promoter (37) and a mutant form of the *Drosophila* HSP70 promoter (R. Kingston, unpublished observations) are not affected. In addition, there is no general increase in the transcription of cellular genes after adenovirus infection. Further, as discussed above, EIa activity does not stimulate transcription from the two initiation sites of EII equally. Thus, some aspect of the mechanism of stimulation must favor particular promoters and initiation sites. These observations are difficult to integrate into a single model for EIa action. One possibility is that most promoters introduced into cells by transfection, microinjection, or infection have a particular rate-limiting step for utilization. The equivalent promoter organized in a natural chromatin setting may not have the same rate-limiting step. The expression of EIa activity in the cell might cause a general change in the transcription apparatus that would result in the stimulation of promoters with this common rate-limiting step. The critical aspect of this model is the suggestion that the common origin of these promoters as exogenously added DNA may be more important in specifying their stimulation by EIa than any specific sequences.

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